Analysis of Mouse Keratin 6a Regulatory Sequences in Transgenic Mice Reveals Constitutive, Tissue-Specific Expression by a Keratin 6a Minigene

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The analysis of keratin 6 expression is complicated by the presence of multiple isoforms that are expressed constitutively in a number of internal stratified epithelia, in palmoplantar epidermis, and in the companion cell layer of the hair follicle. In addition, keratin 6 expression is inducible in interfollicular epidermis and the outer root sheath of the follicle, in response to wounding stimuli, phorbol esters, or retinoic acid. In order to establish the critical regions involved in the regulation of keratin 6a (the dominant isoform in mice), we generated transgenic mice with two different-sized mouse keratin 6a constructs containing either 1.3 kb or 0.12 kb of 5′ flanking sequence linked to the *lacZ* reporter gene. Both constructs also contained the first intron and the 3′ flanking sequence of mouse keratin 6a. Ectopic expression of either transgene was not observed. Double-label immunofluorescence analyses demonstrated expression of the reporter gene in keratin 6 expressing tissues, including the hair follicle, tongue, footpad, and nail bed, showing that both transgenes retained keratinocyte-specific expression. Quantitative analysis of β-galactosidase activity verified that both the 1.3 and 0.12 kb keratin 6a promoter constructs produced similar levels of the reporter. Notably, both constructs were constitutively expressed in the outer root sheath and interfollicular epidermis in the absence of any activating stimulus, suggesting that they lack the regulatory elements that normally silence transcription in these cells. This study has revealed that a keratin 6a minigene contains critical *cis* elements that mediate tissue-specific expression and that the elements regulating keratin 6 induction lie distal to the 1.3 kb promoter region.

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Keratins are the major structural proteins of keratinocytes and form the intermediate filament cytoskeletal network of these cells. More than 30 epithelial keratins have been identified to date and they are classified by their physiochemical properties into two distinct groups. The smaller acidic keratins (K9-K21) are designated as type I keratins whereas the larger, more basic keratins (K1-K8) form the type II keratin group (Moll *et al*, 1982). One member of each type is required to form the heterodimeric coiled-coil precursors that assemble into mature filaments (Hatzfeld and Weber, 1990; Steinert, 1990). The expression of these keratin pairs is coordinated to maintain stoichiometry and is determined by the degree of differentiation and location of the cell. This is most clearly seen in the epidermis where keratin expression switches from keratins K5 (type II) and K14 (type I) in proliferating basal layer cells to K1 and K10 in the differentiating cells of the suprabasal layers (Fuchs and Green, 1980; Moll *et al*, 1982).

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Abbreviations: β-gal, β-galactosidase; CCL, companion cell layer; MK6a, mouse keratin 6a; RA, all-*trans* retinoic acid; X-gal, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside.

In contrast to other keratin genes, K6 and its type I partners (K16 or K17) exhibit both constitutive and inducible regulation (Weiss *et al*, 1984; Stoler *et al*, 1988). K6 is constitutively expressed in the stratified epithelia of palms, soles, nail bed, oral mucosa, tongue, esophagus, forestomach, trachea, reproductive tract, and hair follicles (Moll *et al*, 1982; Quinlan *et al*, 1985; Lynch *et al*, 1986; Rentrop *et al*, 1986; Stark *et al*, 1987; Heid *et al*, 1988a, b). Many of these epithelia exhibit phenotypic changes in pachyonychia congenita patients with K6 mutations (Bowden *et al*, 1995). In normal skin, K6 is only found in the companion cell layer (CCL), a histologically distinct layer located between the inner root sheath and outer root sheath (ORS) of the hair follicle (Rothnagel and Roop, 1995), but is induced in the ORS by activating stimuli (Rothnagel *et al*, 1999). K6 is also induced in interfollicular keratinocytes by mechanical trauma, wounding, and disease states such as psoriasis and skin tumors (Weiss *et al*, 1984; Mannsbridge and Knapp, 1987; Stoler *et al*, 1988). In addition, K6 can be experimentally induced in the epidermis by the topical application of hyperplasia-inducing reagents such as phorbol esters and retinoic acid (Schweizer *et al*, 1987; Rosenthal *et al*, 1992; Heyden *et al*, 1994). The inducible expression of K6 is not limited to hyperproliferating cells but occurs whenever the normal biology of keratinocytes is disturbed, irrespective of the proliferative status of the keratinocyte (Dominey *et al*, 1993; Jiang *et al*, 1993; Sellheyer *et al*, 1993; Imakado *et al*, 1995; Bickenbach *et al*, 1996).

In addition, expression of K6 is induced by placing keratinocytes taken from epidermal, tracheal, and corneal tissues in culture (Weiss *et al*, 1984; Schermer *et al*, 1989; Lindberg and Rheinwald, 1990).

The study of K6 gene regulation and expression is complicated by the occurrence of multiple isoforms encoded by separate genes. Two human K6 genes were initially reported (Tyner *et al*, 1985) but four more paralogs have now been identified (Takahashi *et al*, 1995) and an additional member, termed K6hf, was recently found to be specifically expressed in the CCL of hair follicles (Winter *et al*, 1998). At least three isoforms have been found in bovids (Navarro *et al*, 1995) and two in mice (Takahashi *et al*, 1998; Rothnagel *et al*, 1999). The expression patterns of all of these different isoforms have not been fully described and the contribution of each isoform to overall K6 expression is not yet known. A homology analysis of the two functional mouse K6 genes (denoted MK6a and MK6b) revealed that they share more sequence identity to each other than to the human and bovine K6 genes, suggesting that the duplication events that gave rise to multiple isoforms occurred after species divergence (Takahashi *et al*, 1998). A transgenic analysis of the two mouse paralogs revealed that they had distinct but overlapping patterns of expression (Rothnagel *et al*, 1999).

The regulation and expression of bovine and human K6 genes have recently been investigated in transgenic mice (Ramírez *et al*, 1995, 1998; Takahashi and Coulombe, 1997). A study of the bovine K6β gene found that 9 kb of 5′ flanking sequence was required to provide tissue-specific and inducible expression of a *lacZ* reporter gene in transgenic mice (Ramírez *et al*, 1995). Later it was determined that essential *cis*-acting regulatory sequences for constitutive expression of bovine K6β were located in two distinct regions within this sequence, one distal from (–9.0 kb to –4.0 kb) and one proximal to (–830 to –125 bp) the transcription initiation site (Ramírez *et al*, 1998). By comparison, a human K6a-*lacZ* transgene with 5.2 kb of upstream sequence was unable to drive expression of the reporter in adult tissues that constitutively express endogenous mouse K6. The proximal 960 bp of the human K6a promoter, however, was found to be sufficient to mediate inducible expression, albeit patchy, in response to wounding and hyperplasia-inducing agents (Takahashi and Coulombe, 1997). The different expression characteristics exhibited by these transgenes underscores the need to identify the corresponding mouse orthologs and to investigate the regulation of K6 within each host species.

It was recently shown that a 13.5 kb genomic fragment, containing the entire mouse K6a (MK6a) gene including 6.5 kb of 5′ flanking sequence, exhibited expression in transgenic mice that was indistinguishable from endogenous K6 in all tissues except the tongue (Rothnagel *et al*, 1999). In order to further delineate the sequences involved in regulating MK6a expression, we produced transgenic mice with constructs containing either 1.3 kb or 0.12 kb of 5′ flanking sequences linked to the *Escherichia coli lacZ* reporter gene. We have found that the 0.12 kb MK6a minigene construct contains sufficient sequence information to direct uniform, tissue-specific expression of the reporter gene in transgenic mice. In addition, both the 0.12 and 1.3 kb constructs were constitutively expressed in the ORS and interfollicular epidermis in the absence of an inducing signal.

MATERIALS AND METHODS

DNA constructs and production of transgenic mice The two MK6a promoter/reporter gene constructs used in this study were generated by replacing the coding sequence of a previously isolated 13.5 kb MK6a genomic clone (Rothnagel *et al*, 1999) with a 3 kb *E. coli lacZ* sequence. The constructs contain both 5′ and 3′ flanking sequences, including the untranslated region (UTR) of the K6a mRNA and the first intron, which was retained to ensure efficient transcription. The intron was placed downstream of the 5′ noncoding sequence with the *lacZ* gene cloned between the intron and 3′ noncoding sequences following the organization of the keratinocyte-specific expression vector based on human K1 (Rothnagel *et al*, 1990; Greenhalgh *et al*, 1993). The larger construct (MK6a^{1300}*lacZ*) contains 1.3 kb of 5′ flanking sequence whereas the shorter construct (MK6a^{120}*lacZ*) contains 120 bp of the MK6a promoter (**Fig 1**). The resected transgenes were purified from plasmid sequences by sucrose gradient centrifugation (Mann and McMahon, 1993) and prepared for injection by microconcentration with 10 mM Tris-HCl, 0.1 mM ethylenediamine tetraacetic acid to a final DNA concentration of 2–5 ng per μl. Transgenic mice were produced by pronuclear injection of DNA into single-cell C57BL/CBA embryos essentially as described previously (Hogan *et al*, 1994). Positive mice were identified by polymerase chain reaction (PCR) analysis of genomic tail DNA using *lacZ*-specific primers (forward 5′-TGGTCGTTTTACAACGTC-3′ and reverse 5′-TGTGAG-CGAGTAACAACC-3′) that generate a 356 bp amplicon. Cycling conditions used were 40 cycles at 94°C for 2 min, 56°C for 1 min, and 72°C for 1 min. Another set of primers was used to distinguish between constructs and used an MK6a-specific forward primer (5′-AGATCAAGT-CAAGGCTGGAAGGCA-3′) and a *lacZ*-specific reverse primer (5′-AGG-CGATTAAGTTGGGTAACGCCA-3′). This primer set amplifies an 888 bp product only in transgenic mice containing the larger MK6a^{1300}*lacZ* construct. A control PCR that amplifies a 1440 bp product from the endogenous MK6a gene used the following primers (forward 5′-GCT-CAAACATAGGCTCCAGGTTCT-3′ and reverse 5′-AGATCAAGTC-AAGGCTGGAAGGCA-3′). Transgenic lines were established by matings with BALB/C mice and the F_1 or F_2 offspring were used in all subsequent analyses. Analysis of hair bearing skin was performed in BALB/C animals to avoid auto-fluorescence by the pigmented hair fibres of C57BL mice. Six lines were expanded from the strongest expressors of the MK6a^{1300}*lacZ* founders and four lines from the strongest MK6a^{120}*lacZ* expressors. The initial analysis of expression in back skin and tongue tissues was performed on all lines. Later analysis on whisker, footpad, and nailbed used tissues taken from two lines for each construct.

Histochemical staining of tongue tissue Tongue biopsies (4 mm) were taken, washed briefly in phosphate-buffered saline (PBS), and then incubated at 37°C overnight in 100–150 μl of an X-gal staining solution. This consisted of 100 μg per ml X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) (Progen, Brisbane, Australia) in 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.1% Nonidet P-40, and 0.1 M sodium phosphate, pH 7.7 (Tsuki *et al*, 1996).

RNA extraction and reverse transcriptase (RT)-PCR Total RNA was isolated from approximately 0.05 g of brain, heart, liver, kidney, spleen, and bladder tissue using TRI-Reagent (Molecular Research Center, Cincinnatti, OH). Synthesis of cDNA was performed with 3 μg of total RNA and 200 ng random hexamers (Amersham Pharmacia Biotech, Uppsala, Sweden) using SuperScript II (Gibco/BRL Life Technologies, Gaithersburg, MD) reverse transcriptase according to the manufacturer's instructions. PCR was performed using *lacZ*-specific primers (see above) in a standard reaction (30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C). Primers for mouse *frizzled*-3 were used to amplify a ubiquitous mRNA to verify cDNA synthesis.

Northern hybridization Total skin RNA was isolated from a litter of newborn mice obtained by mating an MK6a^{1300}*lacZ* transgenic male with a BALB/C female. Approximately 80 mg of trunk skin from each littermate was homogenized in TRI-Reagent and RT-PCR was used to identify transgenic animals. Twenty micrograms of RNA was electrophoresed on a denaturing 1% agarose gel containing 1.1% formaldehyde, transferred to Hybond N nylon membrane (Amersham) by capillary diffusion (Sambrook *et al*, 1989), and fixed by ultraviolet radiation. Probes were generated by radio-labeling of PCR amplified MK6a 3′ noncoding and *lacZ* coding sequences. The 230 bp MK6a 3′ noncoding probe was amplified using the following primers: forward 5′-ACCAAGCTTCTGTCACCAAGA-GCT-3′ and reverse 5′-CATGAAGCACCAAATGTG-3′. Twenty-five nanograms of the MK6a PCR product and the 356 bp *lacZ* product (see above) were labeled with α-^{32}PdCTP by random primer labeling using the RTS Radprime kit (Gibco/BRL Life Technologies). The membranes were hybridized at 42°C overnight, washed, and autoradiographed. Northern blots were stripped and then hybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to determine mRNA loading levels. The 356 bp *lacZ* PCR product was cloned into pGEMT-Easy (Promega, Madison, WI) and the 230 bp MK6a 3′ noncoding fragment was cloned into pGEM3Z (Promega) and used to generate cRNA probes for *in situ* hybridization (see below).

***In situ* hybridization** Mouse tissues were immediately excised from animals killed by cervical dislocation and fixed for 4 h in 4% paraformaldehyde in PBS at 4°C, prior to embedding in paraffin. *In situ* hybridization with digoxigenin (DIG)-labeled cRNA probes was performed using a modification of the protocol described by Tohyama *et al* (1994). After deparaffinizing (xylene) and rehydration (ethanol 100%-

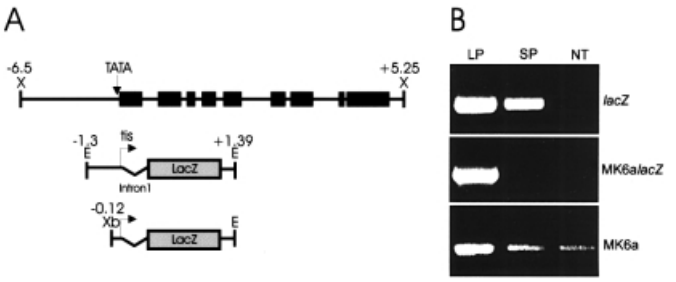

Figure 1. Two truncated MK6a-*lacZ* reporter constructs were used to generate transgenic mice. (*A*) Diagram showing the structure of the MK6a gene and the transgenic constructs used in this study. Nucleotide positions are relative to the transcription initiation site (tis) indicated by the bent arrow. The two different sized MK6a promoter constructs were generated by EcoRI and XbaI digestion, resulting in truncation of the 5′ flanking sequence to 1.3 kb and 0.12 kb, respectively, and excision from the plasmid vector (pGEM 3.2). Both constructs contain the 5′ and 3′ UTRs (62 bp and 535 bp, respectively), the first intron (561 bp), and 3′ flanking (870 bp) sequences of MK6a. The EcoRI, XhoI, and XbaI restriction sites are indicated by E, X, and Xb, respectively. MK6a exons are shown as black boxes. (*B*) PCR of genomic tail DNA samples using two *lacZ*-specific primers amplifies a 356 bp product in mice containing either the MK6a^{1300}*lacZ* (LP) or MK6a^{120}*lacZ* (SP) constructs but not in nontransgenic (NT) control animals (*upper panel*). PCR using an MK6a forward primer that anneals at position –204 bp (5′ of the XbaI site used to generate the SP construct) and a *lacZ* reverse primer amplifies an 888 bp product in MK6a^{1300}*lacZ* transgenic mice but not in MK6a^{120}*lacZ* transgenic or nontransgenic mice (*middle panel*). Successful amplification of a 1440 bp product using primers that are specific for the MK6a gene verified the integrity of the genomic tail DNA samples used (*lower panel*).

70% and PBS) sections were digested for optimal probe penetration with proteinase K (10 μg per ml) for exactly 10 min at 37°C. To minimize nonspecific background signals, sections were acetylated at room temperature for 15 min, and then prehybridized for 2 h at 55°C. The sections were then hybridized with sense or antisense labeled DIG probes at 55°C for 16 h in the presence of 50% formamide, 4 × sodium citrate/chloride buffer (SSC), 1 × Denhardt's, 50% dextran sulfate, and 50 μg per ml yeast tRNA. T7 and Sp6 polymerases were used to generate sense and antisense transcripts labeled with DIG (Roche Molecular Biochemicals, Mannheim, Germany) from the plasmids containing MK6a 3′ noncoding and *lacZ* coding sequences (see above). Probe labeling was determined and concentration was adjusted to ensure that equal amounts of label for each probe were present in the hybridization step. Following hybridization, unbound cRNA probes were removed from the sections by an initial wash with 2 × SSC/50% formamide solution, digestion with 20 μg per ml RNase A at 37°C for 15 min, and a final wash with 1 × SSC/50% formamide at 55°C for 10 min. The sections were then incubated for 30 min at room temperature in 5% normal heat-inactivated sheep serum in 100 mM Tris-150 mM NaCl solution, rinsed, and then incubated for a further 60 min in 0.5% Boehringer blocking reagent in Tris-NaCl at room temperature for 60 min. The bound probes were reacted with anti-DIG antibody conjugated to alkaline phosphatase for 60 min and visualized according to the manufacturer's instructions (Roche Molecular Biochemicals). Endogenous alkaline phosphatase activity was blocked by the addition of freshly prepared levamisole to the color-development solution. Photomicrography was performed with a Zeiss Axiophot Photomicroscope.

Immunofluorescence analysis For immunofluorescence studies tissue samples were dissected and embedded in Tissue Tek II Optimal Cryo Temperature (OCT) compound (Miles, Elkhart, ID) on dry ice (Harlow and Lane, 1988). Sections (8–10 μm) were cut at –20°C, mounted on poly-L-lysine coated slides and allowed to dry for 30 min at room temperature prior to staining. Excess OCT was removed by three 5 min washes in PBS. Blocking was performed in FBT buffer (5% fetal bovine serum, 1% bovine serum albumin, 0.05% Tween 20, 10 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$) for 1 h at room temperature. Primary antibodies used include rabbit anti-*E. coli* β-galactosidase (β-gal) (Cortex, San Leandro, CA, 1:1000 in FBT), rabbit anti-MK6a (Babco, Richmond, CA, 1:500 in FBT), and guinea pig anti-MK6a (1:100 in FBT). The guinea pig MK6a antibodies were generated against the C-terminal peptide sequence (C-KYT-TTSSSKKSYRQ) as described previously (Roop *et al*, 1984). Following overnight incubation at room temperature sections were washed three times in PBS and the secondary antibodies, goat anti-rabbit Alexa 488 conjugate (Molecular Probes, Eugene, OR, 1:500 dilution) and biotinylated goat antiguinea pig (Sigma, St Louis, MO, 1:40 dilution), were applied for 1 h and then washed. The biotinylated guinea pig antibody was detected using Texas Red conjugated to streptavidin (Gibco/BRL, 1:400 dilution). Specimens were mounted in fluorescent mounting medium (Dako, Carpinteria, CA) and imaged with a confocal laser scanning microscope (Biorad MRC 600).

Quantitative determination of β-galactosidase activity Total β-gal activity in tissue extracts was determined using either the Roche Molecular Biochemicals chemiluminescent assay (βgal Reporter Gene kit) or the Biorad (Hercules, CA) fluorescence-based assay (Fluoro-Ace Kit). Ear biopsies (4 mm) from F_0 animals were taken at 0 h and again 24 h later with an overlapping biopsy. Protein extracts were obtained by grinding the samples in liquid nitrogen followed by repeated freeze-thaw cycles according to the manufacturer's instructions. β-gal activity was also determined in protein extracts obtained from back skin, tongue, and whisker tissues of F_1 progeny. Extracts prepared from nontransgenic animals were used to determine control levels. β-gal activity was normalized to total protein content of each extract as determined by a standard colorimetric assay (Bradford, 1976).

Primary keratinocyte culture Primary keratinocytes were harvested from a litter of mice obtained by mating an MK6a^{120}*lacZ* transgenic male with a BALB/C female. β-gal expressing pups were identified by X-gal staining (Tsuki *et al*, 1996) of tongue biopsies. The skins of 2-d-old expressors were incubated in 1.5 ml of 2.5% dispase (Gibco/BRL) at 4°C overnight and the epidermis was separated from the dermis and pooled. Keratinocytes were isolated and cultured as described previously (Jones *et al*, 1997). The keratinocytes were plated at 5×10^5–1×10^6 cells per well in Lab-Tek (Naperville, IL) tissue culture chamber slides (Nunc, Rochester, NY) with 3:1 Dulbecco's modified Eagle's medium:Hams F12 medium (Gibco/BRL) overnight and then in keratinocyte serum-free medium (Gibco/BRL) for 1 d. The culture was washed extensively with PBS before staining with the β-gal antibody as described above.

Topical treatments Hyperproliferative agents were dissolved in acetone and applied topically to the right dorsal flank of shaved mice. The concentrations used were 10 μg per 100 μl of phorbol 12-myristate 13-acetate (PMA, Sigma) and 30 μg per 100 μl of all-*trans* retinoic acid (RA, Sigma). For each animal the same volume of acetone alone was applied to the left flank as a control. Mice were sacrificed at 2, 4, 6, and 24 h post application of PMA and RA. Skin biopsies were taken from each flank and then embedded in OCT for subsequent sectioning and antibody staining.

RESULTS

Generation of MK6a-*lacZ* transgenic mice The MK6a-*lacZ* constructs used in this study are illustrated in **Fig 1**(***A***). Microinjection of the longer construct (MK6a^{1300}*lacZ*), which includes 1.3 kb of the MK6a promoter linked to the *lacZ* gene, into embryos and their subsequent implantation resulted in 107 viable mice, of which 21% were positive for the transgene as determined by PCR using *lacZ*-specific primers. For the short construct (MK6a^{120}*lacZ*), containing 120 bp of proximal promoter sequence, we obtained 93 pups of which 35% harboured the transgene. PCR using the two *lacZ*-specific primers resulted in a 356 bp fragment for transgenic mice harbouring either construct (**Fig 1*B***, *upper panel*). A second PCR was performed using an MK6a forward primer and a *lacZ* reverse primer that specifically amplified an 888 bp product in transgenic mice containing the longer MK6a^{1300}*lacZ* construct. No product was amplified from MK6a^{120}*lacZ* transgenic mouse DNA with these primers (**Fig 1*B***, *middle panel*), demonstrating that the smaller transgene does not contain MK6a promoter sequences 5′ of the XbaI site. Control PCRs using primers specific for the endogenous MK6a gene generated a 1440 bp fragment (**Fig 1*B***, *lower panel*) and confirmed the integrity of the DNA samples. Quantitative comparison of expression levels was performed by chemiluminescent assay of β-gal activity in ear protein extracts. Twenty-two MK6a^{1300}*lacZ* positive mice and 26 MK6a^{120}*lacZ* positive mice expressed the transgene, although animals differed in their level of expression (data not shown). The variation in *lacZ* gene expression was readily apparent after histochemical staining of dissected tongues (**Fig 2**). There was no

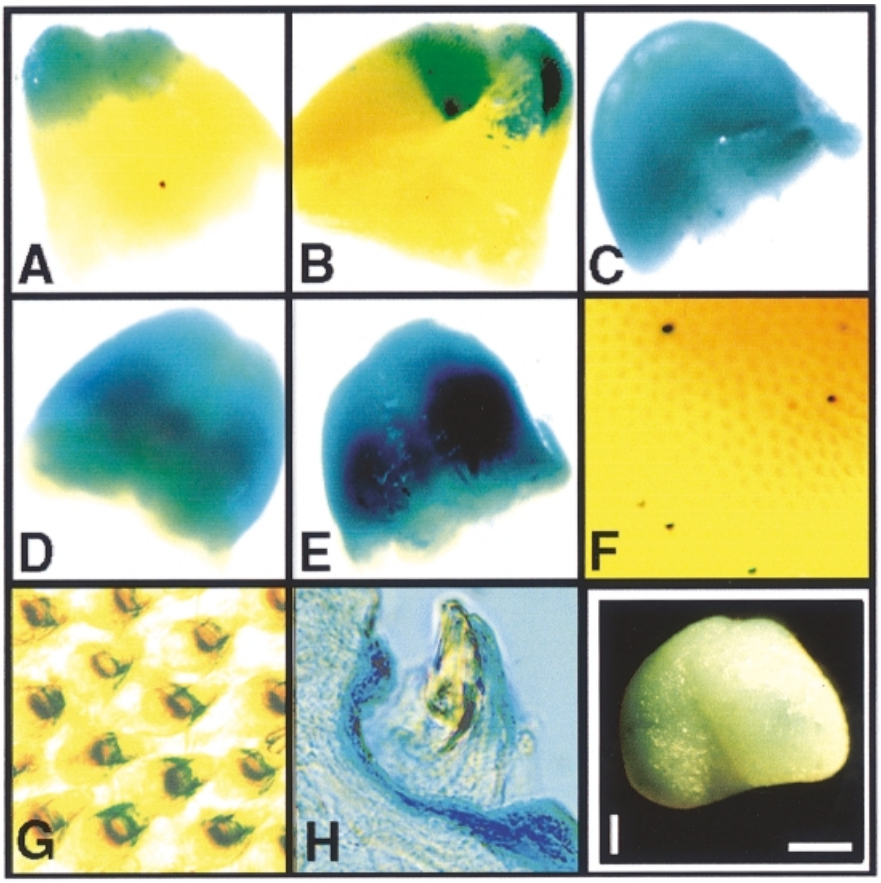

Figure 2. Histochemical staining of tongue biopsies illustrates the variability of β-gal expression observed in different transgenic lines. Staining could be localized to the anterior tip of the tongue (*A*, *B*), throughout the tongue (*C–E*), or to discrete papillae (*F*). At higher magnification X-gal staining is evident in both the papillae and interpapillae epithelium (*G*, *H*). Nontransgenic tongue is negative for X-gal staining (*I*). *Scale bar*: 500 μm (*A-E*, *I*); 125 μm (*F*); 60 μm (*G*); 30 μm (*H*).

correlation between expression levels and copy number as judged by southern analysis on a selection of MK6a^{1300}*lacZ* positive mice that all contained one copy of the transgene (data not shown). The animals exhibiting the strongest expression, as determined by β-gal activity in ear protein extracts, were used for establishment of transgenic lines.

MK6a-*lacZ* transgenes exhibit tissue-specific expression in adult mice RT-PCR analysis of skin and several nonstratified epithelial tissues from MK6a-*lacZ* transgenic animals, including heart, brain, kidney, bladder, spleen, and liver, failed to detect β-gal-specific transcripts in all tissues except skin, confirming that the transgenes were not expressed ectopically (**Fig 3*A***). To localize the MK6a-*lacZ* and endogenous MK6a transcripts, *in situ* hybridization was carried out on normal unstimulated back skin, tongue, and whisker sections from MK6a^{120}*lacZ* mice. The *lacZ* and MK6a DNA templates used to generate DIG-labeled antisense and sense mRNA probes were first tested for specificity by northern analysis using back skin mRNA isolated from both transgenic and nontransgenic animals (**Fig 3*B***). *In situ* hybridization localized the MK6a-*lacZ* transcripts to all living cell layers of tongue (**Fig 4*A***), snout (**Fig 4*B***, ***C***), and back skin (data not shown) epidermis, with strongest expression observed in basal layer keratinocytes. In follicles, transgene expression was localized to ORS keratinocytes (**Fig 4*B***, ***D***). The localization of endogenous MK6a transcripts in hair follicles was similar to that reported previously (Takahashi *et al*, 1998). MK6a transcripts were also detected in interfollicular snout epidermis and in the epithelial cells of the tongue with a distribution similar to the MK6a-*lacZ* mRNAs (data not shown).

A histochemical X-gal staining protocol was initially used to analyze protein expression of the MK6a-*lacZ* transgenes in various K6 expressing tissues. Despite strong staining of β-gal activity in whole tongue biopsies (see **Fig 2**) from both MK6a^{1300}*lacZ* and MK6a^{120}*lacZ* transgenic mice, however, histochemical staining of fresh frozen tongue and back skin sections was minimal and nonuniform, and the blue end-product was difficult to visualize at the cellular level. Weak and inconsistent X-gal staining in keratinocytes had been noted by others who attributed the inadequacies of this methodology to diffusion of the reaction product away from the site of expression (Dunn *et al*, 1998), or instability of β-gal in keratinocytes (Byrne and Fuchs, 1993; Takahashi and Coulombe, 1997). The latter may be a consequence of β-gal inactivation by post-translational processes such as transglutaminase-mediated cross-linking.

To determine whether the observed variability in X-gal staining was a result of poor expression or due to insensitivity of the technique, quantitative fluorometric enzymatic β-gal assays were performed. Soluble extracts were prepared from back skin, whisker, and tongue tissues from MK6a^{1300}*lacZ* and MK6a^{120}*lacZ* transgenic mice. Control extracts from nontransgenic mice were also included. Significant β-gal activity was observed in all three tissues, with levels of enzyme activity 5–20-fold higher than for control extracts (**Fig 5**). Indeed, β-gal activity was higher in back skin and whisker extracts than in tongue. There was no apparent difference in the β-gal activity between extracts prepared from the MK6a^{1300}*lacZ* and MK6a^{120}*lacZ* transgenic mice, indicating that the two constructs are equally efficient at directing keratinocyte-specific expression in transgenic mice. Therefore the apparently low levels of *lacZ* expression observed in follicles using X-gal staining suggest a limitation of the technique rather than lack of β-gal protein expression by these constructs in transgenic skin tissue. In order to overcome this limitation we used an anti-*E. coli* β-gal antibody, which when used in conjunction with a specific MK6a antibody permits the colocalization of transgene expression with endogenous MK6a at the cellular level.

Several K6 expressing tissues from adult mice were analyzed for β-gal localization, including tongue, footpad, nail bed, back skin, and whisker pad. We found that both the MK6a^{1300}*lacZ* and MK6a^{120}*lacZ* transgenes were capable of directing tissue-specific expression of the reporter gene in transgenic mice. Representative examples of antibody staining in tongue are shown in **Fig 4**.

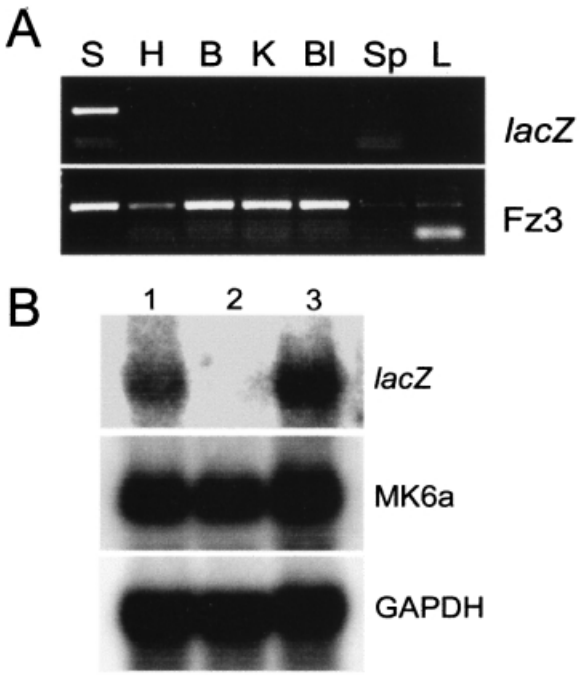

Figure 3. MK6a-*lacZ* transcripts were detected only in skin. (*A*) RT-PCR analysis of selected tissues reveals that the MK6a-*lacZ* transgenes have correct tissue-specific expression. *LacZ* mRNA was detected only in skin and not in nonstratified epithelial tissues from a MK6a^{1300}*lacZ* trangenic mouse including heart (H), brain (B), kidney (K), bladder (B), spleen (Sp), or liver (L) (*upper panel*). PCR reactions with mouse *frizzled*-3 primers amplified a 350 bp product in all tissues, verifying the integrity of the cDNA (*lower panel*). (*B*) The MK6a-*lacZ* transgene and endogenous MK6a transcripts can be detected by northern hybridization. Total RNA was prepared from a newborn litter arising from a mating between an MK6a^{1300}*lacZ* transgenic male and a BALB/C female. *Lanes 1* and *3* contain RNA from two transgenic littermates; *lane 2* contains RNA from a nontransgenic littermate that serves as a negative control. A strong signal for endogenous MK6a was detected in all three mice whereas the *lacZ* transgene was observed only in *lanes 1* and *3*. The signal from the GAPDH probe shows that similar amounts of RNA were present in each lane.

Endogenous MK6a was detected throughout the papillae with both the rabbit and guinea pig anti-MK6a antibodies (**Fig 6*A*, *C*, *D***). As anticipated from the strong X-gal staining observed in whole tongue, both the MK6a^{1300}*lacZ* and MK6a^{120}*lacZ* transgenes were also detected throughout the papillae including noticeable staining of the cornified spines (**Fig 6*E*, *F***), which was readily observable when the single images were merged (**Fig 6*G*, *H***). Furthermore, there was no difference in the pattern of β-gal expression produced by either transgene. Expression of both endogenous MK6a and the transgenes was found to be greatest in the papillary and interpapillary epithelium at the anterior tip of the tongue, with staining intensity decreasing distally. A similar gradient of K6 transgene expression in the tongue was previously observed in a study using a bovine K6-*lacZ* construct (Ramírez *et al*, 1995).

In footpad epidermis, endogenous MK6a detected with the rabbit antibody was found in both basal and suprabasal keratinocytes (**Fig 7*A***), whereas the guinea pig antibody predominantly stained the basal layer (**Fig 7*B***). As both antibodies were generated using the same antigen and endogenous MK6a transcripts have been detected in both basal and suprabasal cells by *in situ* hybridization (Takahashi *et al*, 1998), the differential staining by these antibodies suggests differences in epitope recognition. Both the MK6a^{1300}*lacZ* and MK6a^{120}*lacZ* constructs directed expression of β-gal to all cell layers of footpad epidermis (**Fig 7*C*, *D*, *E***) reflecting the expression of the native gene with no differences seen between the two transgenes. Similarly, β-gal staining of nail bed sections showed that both MK6a^{1300}*lacZ* and MK6a^{120}*lacZ* transgenes were expressed uniformly throughout the epithelium, whereas endogenous guinea pig anti-MK6a staining was more apparent in basal layer keratinocytes (**Fig 7*F*, *G***).

MK6a-*lacZ* transgenes are constitutively expressed in interfollicular and ORS keratinocytes Although both of the MK6a-*lacZ* transgenes exhibited tissue specificity, their expression did not always coincide with that of the endogenous MK6a gene. This was clearly evident in back skin biopsies where β-gal expression but not endogenous MK6a (using either rabbit or guinea pig MK6a antibodies) was observed in the interfollicular keratinocytes of unstimulated epidermis (**Fig 8*A***). In the hair follicle, we consistently observed β-gal expression in the CCL and ORS keratinocytes in both MK6a^{1300}*lacZ* and MK6a^{120}*lacZ* transgenic mice, even in the absence of an activating stimulus (**Fig 8*A*, *C***). This is in contrast to endogenous MK6 expression, which is normally limited to the CCL but expands into the ORS upon activation (Rothnagel *et al*, 1999). The expression pattern in snout skin was similar to that observed in back skin with both transgenes expressed in whisker follicles and throughout the interfollicular snout epidermis. The expression of both of the MK6a-*lacZ* transgenes and endogenous MK6a appeared to be higher in snout skin than in back skin as indicated by the stronger signal produced by their respective antibodies (**Fig 8*B***) and is consistent with the findings of an earlier transgenic study (Takahashi and Coulombe, 1997). These authors postulated that increased levels of K6 resulted from a ''constant induction'' of K6 in whisker follicles as a consequence of the continual rubbing of this area during grooming. Antibody staining of the large vibrissae follicles of the snout show colocalization of β-gal with endogenous MK6a in both the CCL and ORS (inset, **Fig 8*B***). We also noted that endogenous MK6a was usually present in the interfollicular epidermis of the snout as well (**Fig 8*B***), an observation that to our knowledge has not been previously reported.

To determine whether hyperproliferative agents could alter the expression of the MK6a-*lacZ* transgenes, RA and PMA were applied topically to the shaved back skins of transgenic littermates. Animals were sacrificed and back skin biopsies were taken at several time points (0, 2, 4, 6, and 24 h after application) to allow a comparison of the kinetics of any induced response. Induction of endogenous K6a expression from the CCL into the ORS and interfollicular epidermis was already evident at the 2 h time point and persisted for at least 24 h post application (compare noninduced skin in **Fig 8*C*** with **Fig 8*D*, *E***). This concurs with previous studies that found that MK6a was induced 1 h after treatment with PMA, reaching peak expression by 12 h and persisting for at least 96 h (Heyden *et al*, 1994; Takahashi *et al*, 1998). No changes in the pattern of β-gal staining were observed in our transgenic mice, however, and as these transgenes are expressed constitutively in the epidermis of these animals, any increase in interfollicular expression may not be readily observable by immunofluorescence analysis. We therefore attempted to assess any changes in β-gal expression by measuring enzyme activity but this proved not to be possible as the RA and PMA treated extracts inhibited the assay.

MK6a-*lacZ* transgenes are constitutively expressed in cultured keratinocytes A number of studies have shown that K6 is expressed in cultured keratinocytes (Weiss *et al*, 1984; Schermer *et al*, 1989; Lindberg and Rheinwald, 1990). We therefore determined whether β-gal expression occurred in cultured primary keratinocytes harvested from the skins of an F_1 MK6a^{120}*lacZ* litter. Although there was variability in the level of staining, all cells were positive for β-gal (**Fig 8*F***). Taken together with the immunofluorescence analysis these results suggest that truncation of the MK6a promoter to 120 bp does not abrogate expression of the *lacZ* gene in transgenic mice.

DISCUSSION

The starting point for this study was the observation that a 13.5 kb fragment containing the complete MK6a gene, including 6.5 kb of 5′ flanking sequence, showed tissue-specific constitutive and

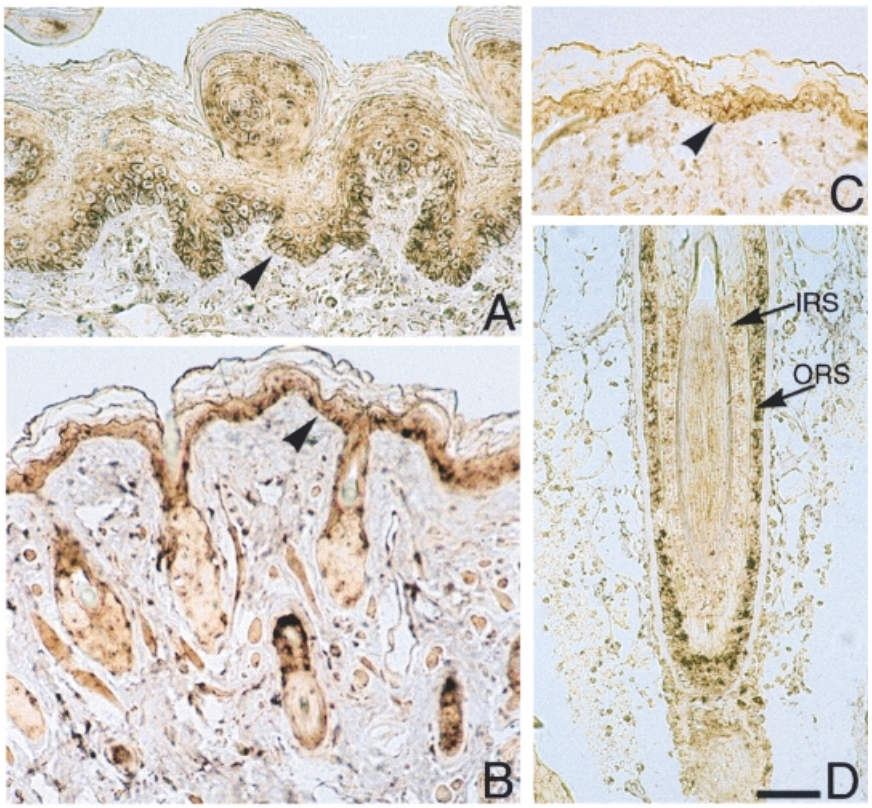

Figure 4. *In situ* hybridization using a β-gal-specific mRNA probe shows that the MK6a^{120}*lacZ* transcript is expressed in K6 expressing tissues. In tongue sections (*A*) expression was observed in all layers of the papillary epidermis but is strongest in the basal layer keratinocytes. Similarly, β-gal mRNA is detected in all layers of snout epidermis (*B*, *C*). The *arrowheads* (*A*, *B*, and *C*) indicate the dermal-epidermal junction. A longitudinal section of a whisker follicle (*D*) shows that MK6a^{120}*lacZ* transcripts are present in both the CCL and ORS. *Scale bar*: 50 μm (*A–C*); 100 μm (*D*).

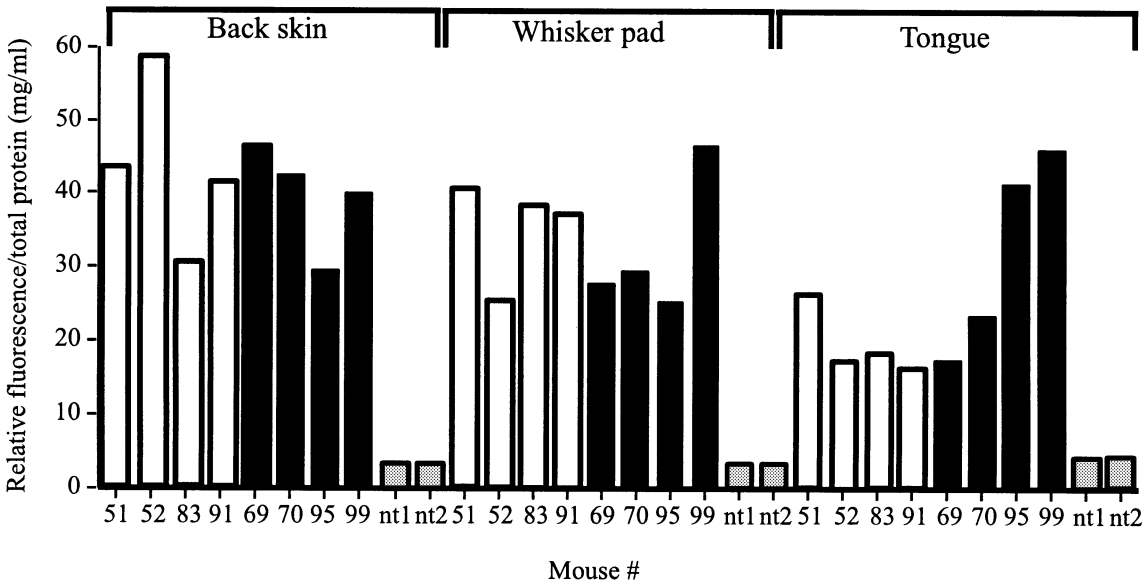

Figure 5. Quantitative βgal activity assay of several K6-expressing tissues from MK6a^{1300}*lacZ* and MK6a^{120}*lacZ* transgenic mice. Back skin, whisker and tongue tissues of two F_1 littermates from two different lines of MK6a^{1300}*lacZ* transgenic mice (open bars) and MK6a^{120}*lacZ* transgenic mice (solid bars) were analysed and compared to non-transgenic (nt) control animals (hatched bars). Relative fluorescence activity was normalised to the total protein content of each extract.

inducible expression in transgenic mice (Rothnagel *et al*, 1999). In order to define the minimal sequences required for regulation of MK6a expression, we analyzed the ability of two constructs containing either 1.3 or 0.12 kb of the MK6a promoter, the first intron, and 3′ noncoding sequences to drive expression of the *lacZ* reporter gene in transgenic mice. Surprisingly, our analyses revealed that both constructs were capable of directing sustained, uniform expression of the reporter in K6 expressing tissues including skin, whisker pad, tongue, nail bed, and footpad epithelia. Aberrant expression of the transgenes in non-K6 expressing tissues was not observed. Furthermore, there was no discernible difference between the MK6a^{1300}*lacZ* and MK6a^{120}*lacZ* transgenes in either their patterns or levels of expression.

It is noteworthy that all transgenic animals expressed β-gal to some degree and that more than 20% showed strong expression based on tongue and ear biopsies. Only a few founder animals showed sporadic, nonuniform expression. The level of transgene expression was an inheritable trait and β-gal levels of F_1 and F_2

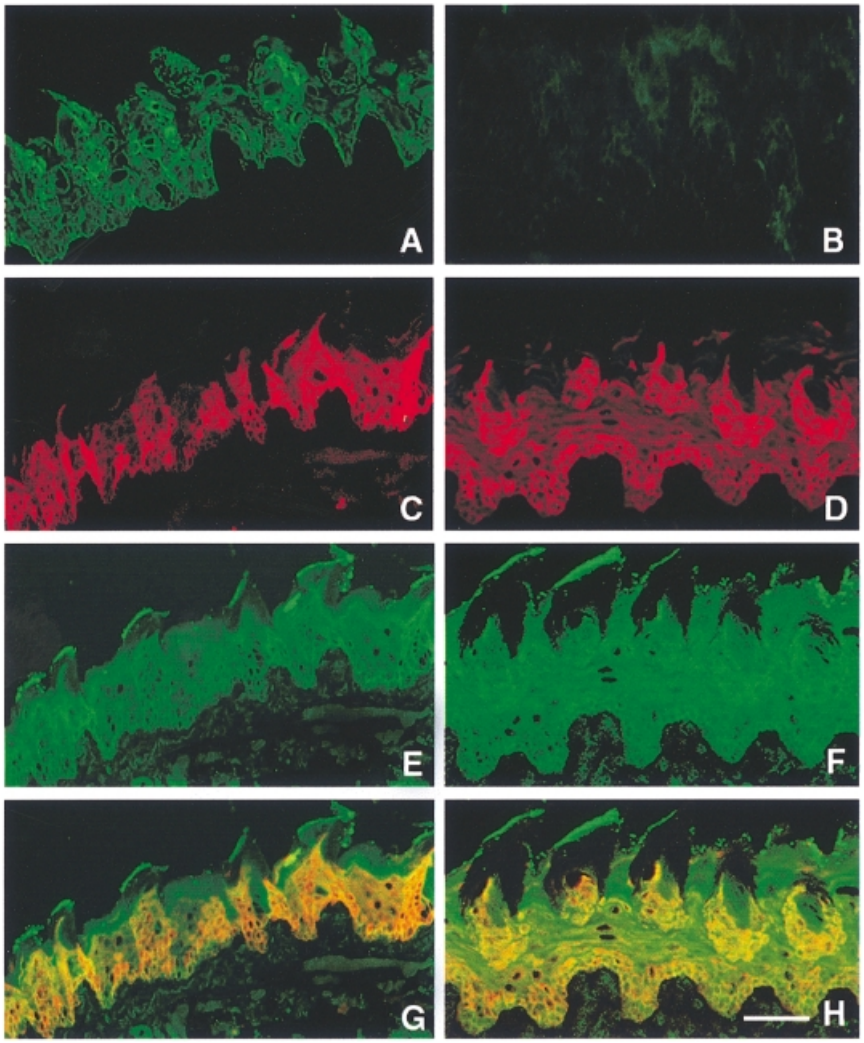

Figure 6. Immunofluorescence antibody staining of tongue sections reveals efficient expression of the MK6a-*lacZ* transgenes that mimics that of the endogenous MK6a gene. The endogenous MK6a protein was localized throughout the tongue papillae with either the rabbit (*A*) or guinea pig (*C*, *D*) primary antibodies. Anti-β-gal staining was not detected in nontransgenic tissues (*B*) or in transgenic tissues where the primary antibody was omitted (not shown). The anti-β-gal staining patterns in tongue sections from MK6a^{1300}*lacZ* (*E*) and MK6a^{120}*lacZ* (*F*) transgenic mice were identical and colocalized with the endogenous protein, except for the very tip of the cornified spines of the papillae (*G*, *H*). *Scale bar*: 80 μm (*A*, *B*, *D*, *F*, *H*); 100 μm (*C*, *E*, *G*).

progeny were similar to that of their founders. Previous transgenic studies using truncated keratin promoters to drive *lacZ* gene expression have reported a low percentage of animals expressing the transgene, presumably due to greater sensitivity of short promoters to positional effects (Byrne and Fuchs, 1993; Dunn *et al*, 1998). Positional effects can also cause variegation of transgene expression because of chromatin influences on regulatory regions at or near the site of integration that can affect both the level and specificity of transgene expression (Dillon and Grosveld, 1993; Martin and Whitelaw, 1996). As insertion of transgenic DNA into the genome occurs at random sites and given that we generated several independent transgenic founders, our results suggest that the regulatory sequences within the MK6a constructs are largely (but not absolutely) insulated from chromatin influences. A similar observation was made for the human K8 and K18 promoters where an *Alu* repetitive sequence was postulated to insulate these promoters from elements lying outside the transgene (Thorey *et al*, 1993; Casanova *et al*, 1995). Whether an analogous insulating sequence exists in the MK6a constructs remains to be determined.

The uniform expression of the MK6a promoter constructs observed in this study is in contrast to previous studies in which sporadic nonuniform expression of human and bovine K6-*lacZ* constructs was observed even with relatively long 5′ flanking sequences (Ramírez *et al*, 1995; Takahashi and Coulombe, 1997). The patchy expression of the human and bovine transgenes may be due to these constructs lacking critical *cis* elements or may reflect differences in the regulation of K6 orthologs. Alternatively, the different expression profiles observed with the bovine, human, and mouse K6-*lacZ* transgenes may be attributable to the assay itself. It has been noted that X-gal staining can produce false negatives, as the method relies on a threshold level of functional enzyme to produce a visible product (Fire, 1992). In these studies we found patchy, nonuniform X-gal staining of hair follicles in back skin, yet uniform staining of this tissue using a specific antibody. Moreover, substantial β-gal enzyme activity could be detected in tissue extracts of transgenic back skin using a fluorometric assay. This suggests that either the X-gal substrate failed to reach cells expressing β-gal or that enzyme levels in these keratinocytes were below that required for histochemical detection.

A major difficulty in comparing the results of the human and bovine transgenic studies with this study is that it is not possible to unequivocally assign the human K6a and bovine K6β genes as functional orthologs of MK6a. A determination based on sequence homology comparisons is not definitive because the degree of divergence between K6 paralogs is less than that between the K6 genes of different species (Takahashi *et al*, 1998; Rothnagel *et al*, 1999). A comparison of the expression profiles of the human and bovine K6 transgenes shows some overlap with MK6a but also distinct differences. The bovine K6β transgene was expressed constitutively in the CCL of the hair follicle, the papilla of the tongue, in several internal epithelial tissues, and in primary cultures of transgenic epidermis (Ramírez *et al*, 1995). Notably, the bovine transgene was expressed suprabasally in stratified epithelia and in the interfollicular epidermis after induction. The human K6a gene was only weakly expressed in the CCL and not at all in the tongue but exhibited suprabasal expression in wounded or PMA treated skin

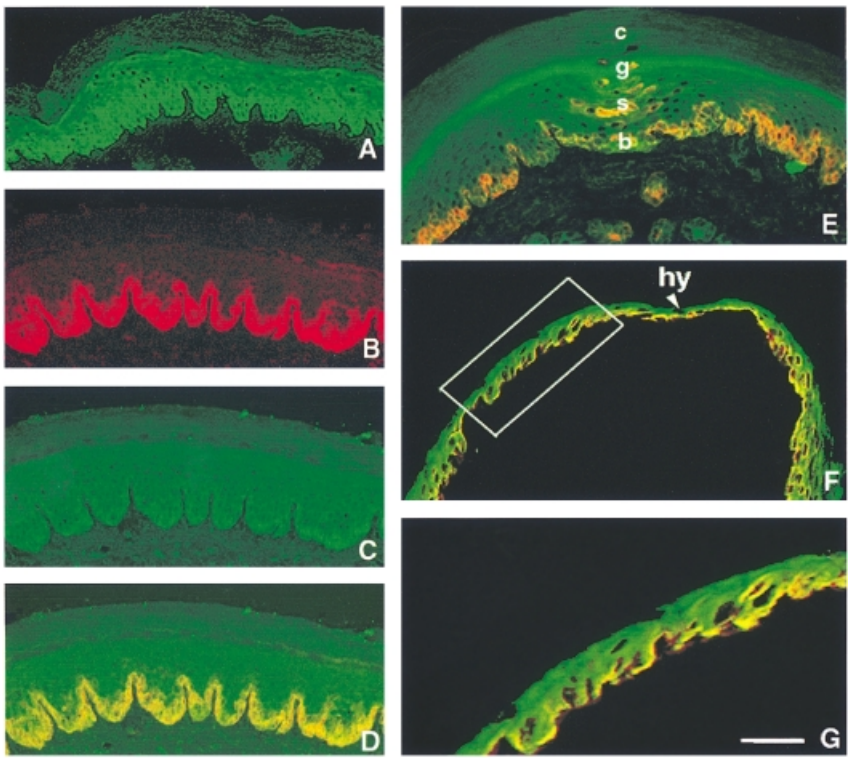

Figure 7. Immunofluorescence antibody staining shows MK6a-*lacZ* transgenes are also expressed in footpad and nail bed epithelium. The rabbit MK6a antibody detected endogenous K6a in all epidermal layers (*A*), whereas the guinea pig primary antibody detected MK6a predominantly in basal layer keratinocytes (*B*). β-gal expression from the MK6a^{120}*lacZ* (D - merged image of *B* and *C*) and MK6a^{1300}*lacZ* (*E*) transgenes was detected in all epidermal layers; indicated in *panel E* as the basal (b), spinous (s), granular (g), and cornified (c) layers. Similar basal keratinocyte colocalization was observed in nail bed. In (*F*), the hyponychium (hy) of the nail bed is indicated and the boxed area is shown at higher magnification in *G*. *Scale bar*: 200 μm (*A–D*); 100 μm (*E*); 50 μm (*F*), and 30 μm (*G*).

(Takahashi and Coulombe, 1997). The mainly suprabasal expression of the bovine and human transgenes is consistent with their native expression patterns and suggests that they are equivalent to the suprabasally expressed MK6b gene (Takahashi *et al*, 1998; Rothnagel *et al*, 1999) rather than orthologs of MK6a. It remains to be determined if any of the other bovine and human K6 genes are orthologs of the MK6a gene or if the basal cell expression of MK6a is unique to rodents.

Although we did not observe ectopic expression of the transgenes in nonkeratinizing tissues, we consistently observed uniform expression of the reporter in keratinocytes of the interfollicular epidermis and ORS, even in the absence of any apparent stimulus. This compares to endogenous K6 expression, which is expressed at high levels in the CCL of control (nonactivated) back skin but not in the ORS and interfollicular epidermis until induced by RA, PMA, or wounding stimuli. We did, however, observe K6 staining in the interfollicular epidermis of some sections from apparently normal, unstimulated back skin. This staining was always of a much lower intensity than the fluorescent signal observed in the CCL of follicles but nonetheless detectable by confocal microscopy. The antibody staining was corroborated by the *in situ* hybridization assay, which detected low levels of endogenous K6a transcripts in this tissue. Whether this expression reflects low level constitutive expression or induction from grooming is not known.

The constitutive expression of the MK6a-*lacZ* transgenes in the epidermis and ORS indicates that these constructs lack the *cis*-regulatory elements required to silence K6a expression in this tissue. This observation suggests that K6 induction is mediated through the removal of negative factors rather than by the addition of positive regulators. We have shown previously that expression of a 13.5 kb MK6a transgene (containing 6.5 kb of 5′ flanking sequence, introns 1–6, and the 3′ noncoding sequence) was limited to the CCL in unstimulated epidermis (Rothnagel *et al*, 1999). In addition, we have recently generated transgenic mice expressing an MK6a-*Cre* transgene with 6.5 kb of MK6a 5′ flanking sequences (as well as intron 1 and the 3′ noncoding and flanking sequences) linked to the bacteriophage P1 *Cre* recombinase gene. These mice show faithful follicle-specific expression of *Cre* in the CCL of normal unstimulated skin and not in the ORS or interfollicular epidermis prior to induction, but can be induced in these cells in response to RA and PMA treatments (Smyth *et al*, in preparation). Therefore the putative *cis* elements that restrict MK6a expression to the CCL and mediate induction in the ORS and interfollicular epidermis must lie between –6.5 and –1.3 kb, as these sequences are not present in the MK6a-*lacZ* constructs. By comparison, the sequences that mediate induction of the human and bovine K6 genes in transgenic mice have been mapped to –0.96 and –0.55 kb, and between –2.4 and –0.83 kb, respectively (Takahashi and Coulombe, 1997; Ramírez *et al*, 1998). If these are the true orthologs of MK6a, then there is a marked difference in the arrangement of promoter elements across the different species.

This study has shown that a mouse K6a minigene can direct uniform tissue-specific expression in transgenic animals and that regulatory elements that determine keratinocyte specificity lie within the 120 bp of the proximal promoter or within the first intron and 3′ flanking sequences of these constructs. Systematic analyses of other keratin genes suggest that tissue specificity is most probably conferred by sequences proximal to the TATA box. A transgenic analysis of the human K5 promoter revealed that 90 bp of 5′ flanking sequences contained sufficient information to direct expression to keratinocytes (Byrne and Fuchs, 1993). Indeed a number of transgenic studies have shown the retention of cell-type specificity of truncated keratinocyte-specific promoters (Chung *et al*, 1994; DiSepio *et al*, 1995; Crish *et al*, 1998; Dunn *et al*, 1998). An analysis of the human K6 promoter in transfected cells showed that 390 bp of 5′ sequences could direct expression of a reporter gene in keratinocytes but not in fibroblasts (Jiang *et al*, 1991). A similar observation was made for the bovine K6β promoter (Blessing *et al*, 1989). A detailed analysis of this region found that the sequences proximal to the TATA box are highly conserved with the mouse and human K6 genes, although it appears that nuclear factor binding to this region differs significantly between the human and bovine genes (Bernerd *et al*, 1993; Navarro *et al*, 1995). An *in vitro* analysis of the MK6a promoter will be necessary to determine whether these sites are functional in the mouse gene and to ascertain their role in cell-type specificity.

In summary, we have found that a MK6a minigene gives uniform, tissue-specific expression of a heterologous reporter in transgenic mice. Expression vectors based on this MK6a promoter

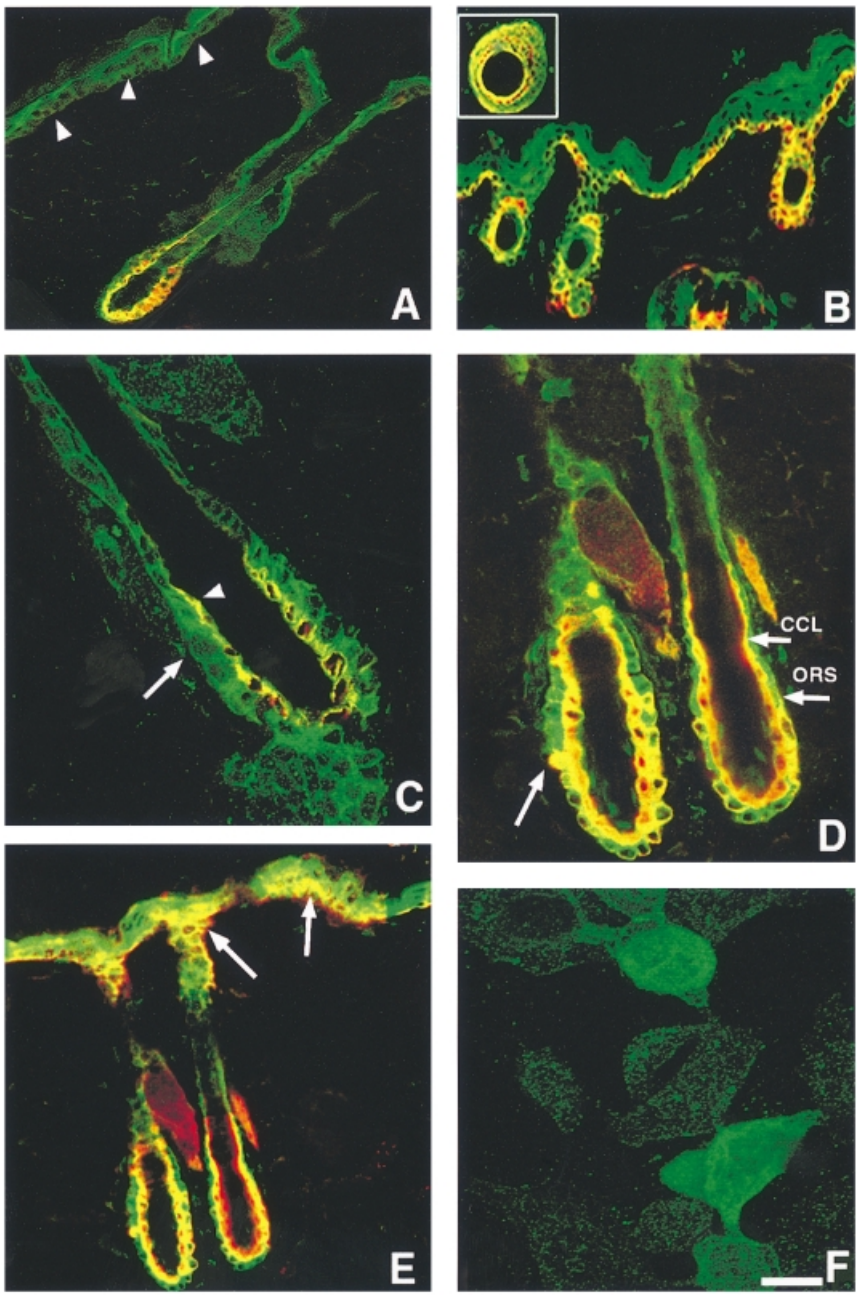

Figure 8. Double-label immunofluorescence antibody staining of adult back skin for endogenous MK6a and transgenic expression in normal and RA treated back skin. In normal skin (*A*) anti-β-gal staining is present throughout the interfollicular epidermis and in the CCL and ORS of hair follicles. *Arrowheads* demarcate the basement membrane. In whisker pad skin (*B*), both β-gal and MK6a staining is observed in the interfollicular epidermis and in the CCL and ORS of vibrissae follicles. The *inset* shows colocalization of β-gal and MK6a proteins in the CCL and ORS of a large, sensory vibrissae follicle in transverse section. In control skin (*C*), β-gal is present in the CCL and the ORS (*arrow*) whereas endogenous MK6a expression is limited to the CCL (*arrowhead*). In treated skin (*D*, *E*), 24 h after a single application of RA, MK6a expression is induced in ORS cells (*D*, *arrow*) and in interfollicular epidermal keratinocytes (*E*, *arrows*). Anti-β-gal staining shows transgene expression in cultured primary keratinocytes (*F*) isolated from mice containing the MK6a^{120}*lacZ* construct. *Scale bar*: 100 μm (*A*, *B*); 30 μm (*C–E*) and 10 μm (*F*).

construct may therefore prove useful for targeting the expression of exogenous proteins to the skin, with potential applications in gene-based therapies for the amelioration of skin disorders or in transgenic studies examining skin biology.

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REFERENCES

Bernerd F, Magnaldo T, Freedberg IM, Blumenberg M: Expression of the carcinoma-associated keratin K6 and the role of AP-1 proto-oncoproteins. *Gene Expression* 3:187–199, 1993

Bickenbach JR, Longley MA, Bundman DS, Bowden PE, Rothnagel JA, Roop DR: A transgenic model recapitulates the clinical features of both neonatal and adult forms of the skin disease epidermolytic hyperkeratosis. *Differentiation* 61:129–139, 1996

Blessing M, JorcanoJL, Franke WW: Enhancer elements directing cell-type-specific expression of cytokeratin genes and changes of the epithelial cytoskeleton by transfections of hybrid cytokeratin genes. *EMBO J* 8:117–126, 1989

Bowden PE, Haley JL, Kansky A, Rothnagel JA, Jones DO, Turner RJ: Mutation of a type II keratin gene (K6a) in pachyonychia congenita. *Nature Genet* 10:363–365, 1995

Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 7:248–254, 1976

Byrne C, Fuchs E: Probing keratinocyte and differentiation specificity of the human K5 promoter *in vitro* and in transgenic mice. *Mol Cell Biol* 13:3176–3190, 1993

Casanova L, Bravo A, Were F, Ramírez A, Jorcano JJ, Vidal M: Tissue-specific and efficient expression of the human simple epithelial keratin 8 gene in transgenic mice. *J Cell Sci* 108:811–820, 1995

Chung S, Cheng CK, Rothnagel JA, *et al*: Expression of the human keratin gene (K1) in transgenic mice is tissue- and developmental-specific but altered with respect to differentiation state. *Mol Cell Diff* 2:61–81, 1994

Crish JF, Zaim TM, Eckert RL: The distal regulatory region of the human involucrin promoter is required for expression in epidermis. *J Biol Chem* 273:30460–30465, 1998

Dillon N, Grosveld F: Transcriptional regulation of multigene loci: multilevel control. *Trends Genet* 9:134–137, 1993

DiSepio D, Jones A, Longley MA, Bundman D, Rothnagel JA, Roop DR: The proximal promoter of the mouse loricrin gene contains a functional AP-1 element and directs keratinocyte-specific but not differentiation-specific expression. *J Biol Chem* 270:10792–10799, 1995

Dominey AM, Wang XJ, King LE Jr, *et al*: Targeted overexpression of transforming growth factor alpha in the epidermis of transgenic mice elicits hyperplasia hyperkeratosis, and spontaneous, squamous papillomas. *Cell Growth Differ* 4:1071–1082, 1993

Dunn SM, Keough RA, Rogers GE, Powell BC: Regulation of a hair follicle keratin intermediate filament gene promoter. *J Cell Science* 111:3487–3496, 1998

Fire A: Histochemical techniques for locating *Escherichia coli* β-galactosidase activity in transgenic organisms. *Genet Anal Tec Appl* 9:151–158, 1992

Fuchs E, Green H: Changes in keratin gene expression during terminal differentiation of the keratinocyte. *Cell* 19:1033–1042, 1980

Greenhalgh DA, Rothnagel JA, Wang X-J, *et al*: Hyperplasia, hyperkeratosis and benign tumor production in transgenic mice by a targeted v-fos oncogene suggest a role for fos in epidermal differentiation and neoplasia. *Oncogene* 8:2145–2157, 1993

Harlow E, Lane D: *Antibodies: a Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1988

Hatzfeld M, Weber K: The coiled coil of *in vitro* assembled keratin filaments is a heterodimer of type I and type II keratins: use of site-specific mutagenesis and recombinant protein. *J Cell Biol* 110:1199–1210, 1990

Heid HW, Moll I, Franke WW: Patterns of expression of trichocytic and epithelial cytokeratins in mammalian tissues. I. Human and bovine hair follicles. *Differentiation* 37:137–157, 1988a

Heid HW, Moll I, Franke WW: Patterns of expression of trichocytic and epithelial cytokeratins in mammalian tissues. II. Concomitant and mutually exclusive synthesis of trichocytic and epithelial cytokeratins in diverse human and bovine tissues (hair follicle, nail bed and matrix, lingual papilla, thymic reticulum). *Differentiation* 37:215–230, 1988b

Heyden A, Lützow-Holm C, Clausen OPF, Brandtzaeg P, Huitfeldt HS: Expression of keratins K6 and K16 in regenerating mouse epidermis is less restricted by cell replication than the expression of K1 and K10. *Epith Cell Biol* 3:96–101, 1994

Hogan B, Beddington R, Constantini F, Lacy E: *Manipulating the Mouse Embryo: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1994

Imakado S, Bickenbach JR, Bundman DS, *et al*: Targeting expression of a dominant-negative retinoic acid receptor mutant in the epidermis of transgenic mice results in loss of barrier function. *Genes Dev* 9:317–329, 1995

Jiang C-K, Epstein HS, Tomic M, Freedberg IM, Blumenberg M: Functional comparison of the upstream regulatory DNA sequences of four human epidermal keratin genes. *J Invest Dermatol* 96:162–167, 1991

Jiang C-K, Magnaldo T, Ohtsuki M, Freedberg IM, Bernerd F, Blumenberg M: Epidermal growth factor and transforming growth factor specifically induce the activation- and hyperproliferation-associated keratins 6 and 16. *Proc Nat Acad Sci USA* 90:6786–6790, 1993

Jones SJ, Dicker AJ, Dahler AL, Saunders NA: E2F as a regulator of keratinocyte proliferation: implications for skin tumor development. *J Invest Dermatol* 109:187–193, 1997

Lindberg K, Rheinwald JG: Three distinct keratinocyte subtypes identified in human oral epithelium by their patterns of keratin expression in culture and in xenografts. *Differentiation* 45:230–241, 1990

Lynch MH, O'Guin WM, Hardy C, Mak L, Sun T-T: Acidic and basic hair/nail ('hard') keratins: their colocalization in upper cortical and cuticle cells of the human hair follicle and their relationship to 'soft' keratins. *J Cell Biol* 103:2593–2606, 1986

Mann JR, McMahon AP: Factors influencing frequency production of transgenic mice. *Meth Enzymol* 225:771–781, 1993

Mannsbridge JN, Knapp AM: Changes in keratinocyte maturation during wound healing. *J Invest Dermatol* 89:253–263, 1987

Martin DI, Whitelaw E: The vagaries of variegated transgenes. *Bioessays* 18:919–923, 1996

Moll R, Franke WW, Schiller DL, Geiger B, Krepler R: The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* 31:11–24, 1982

Navarro JM, Casatorres J, Jorcano JL: Elements controlling the expression and induction of the skin hyperproliferation-associated keratin K6. *J Biol Chem* 270:21362–21367, 1995

Quinlan RA, Schiller DL, Hatzfeld M, *et al*: Patterns of expression and organization of cytokeratin intermediate filaments. *Ann N Y Acad Sci* 455:282–306, 1985

Ramírez A, Vidal M, Bravo A, Larcher F, Jorcano JL: A 5′-upstream region of a bovine keratin 6 gene confers tissue-specific expression and hyperproliferation-related induction in transgenic mice. *Proc Nat Acad Sci USA* 92:4783–4787, 1995

Ramírez A, Vidal M, Bravo A, Jorcano JL: Analysis of sequences controlling tissue-specific and hyperproliferation-related keratin 6 expression in transgenic mice. *DNA Cell Biol* 17:177–185, 1998

Rentrop M, Knapp B, Winter H, Schweizer J: Differential localization of distinct keratin mRNA-species in mouse tongue epithelium by *in situ* hybridization with specific cDNA probes. *J Cell Biol* 103:2583–2591, 1986

Roop DR, Cheng CK, Titterington L, Meyers CA, Stanley JR, Steinert PM, Yuspa SH: Synthetic peptides corresponding to keratin subunits elicit highly specific antibodies. *J Biol Chem* 259:8037–8040, 1984

Rosenthal DS, Griffiths CEM, Yuspa SH, Roop DR, Voorhees JJ: Acute or chronic topical retinoic acid treatment of human skin *in vivo* alters the gene expression of epidermal transglutaminase, loricrin, involucrin, filaggrin and keratins 6 and 13 but not keratins 1, 10, and 14. *J Invest Dermatol* 98:343–350, 1992

Rothnagel JA, Roop DR: Hair follicle companion layer: reacquainting an old friend. *J Invest Dermatol* 104:42S–43S, 1995

Rothnagel JA, Longley MA, Bundman D, Greenhalgh DA, Dominey AM, Roop DR: Targeting gene expression to the epidermis of transgenic mice: potential applications to genetic skin disorders. *J Invest Dermatol* 95:59S–61S, 1990

Rothnagel JA, Seki T, Ogo M, *et al*: The mouse keratin 6 isoforms are differentially expressed in the hair follicle, footpad, tongue and activated epidermis. *Differentiation* 65:119–130, 1999

Sambrook J, Fritsch E, Maniatis T: *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1989

Schermer A, Jester JV, Hardy C, Milano D, Sun T-T: Transient synthesis of K6 and K16 keratins in regenerating rabbit corneal epithelium, keratin markers for an alternative pathway of keratinocyte differentiation. *Differentiation* 42:103–110, 1989

Schweizer J, Furstenberger G, Winter H: Selective suppression of two postnatally acquired 70kD and 65kD keratin proteins during continuous treatment of adult mouse tail epidermis with vitamin A. *J Invest Dermatol* 89:125–131, 1987

Sellheyer K, Bickenbach JR, Rothnagel JA, *et al*: Inhibition of skin development by over-expression of transforming growth factor beta 1 in the epidermis of transgenic mice. *Proc Nat Acad Sci USA* 90:5237–5241, 1993

Stark HJ, Breitkreutz D, Limat A, Bowden PE, Fusenig NE: Keratins of the human hair follicle: 'hyperproliferative' keratins consistently expressed in outer root sheath cells *in vivo* and *in vitro*. *Differentiation* 35:236–248, 1987

Steinert PM: The two-chain coiled-coil molecule of native epidermal keratin intermediate filaments is a type I-type II heterodimer. *J Biol Chem* 265:8766–8774, 1990

Stoler A, Kopan R, Duvic M, Fuchs E: Use of monospecific antisera and cRNA probes to localize the major changes in keratin expression during normal and abnormal epidermal differentiation. *J Cell Biol* 107:427–446, 1988

Takahashi K, Coulombe PA: Defining a region of the human keratin 6a gene that confers inducible expression in stratified epithelia of transgenic mice. *J Biol Chem* 272:11979–11985, 1997

Takahashi K, Paladini RD, Coulombe PA: Cloning and characterization of multiple human genes and cDNAs encoding highly related type II keratin 6 isoforms. *J Biol Chem* 270:18581–18592, 1995

Takahashi K, Yan B, Yamanishi K, Imamura S, Coulombe P: The two functional keratin 6 genes of mouse are differentially regulated and evolved independently from their human orthologs. *Genomics* 53:170–183, 1998

Thorey IS, Ceceña G, Reynolds W, Oshima RG: *Alu* sequence involvement in transcriptional insulation of the keratin 18 gene in transgenic mice. *Mol Cell Biol* 13:6742–6751, 1993

Tohyama C, Nishimura N, Suzuki JS, Karasawa M, Nishimura H: Metallothionein mRNA in the testis and prostate of the rat detected by digoxigenin-labelled riboprobe. *Histochemistry* 101:341–346, 1994

Tsuki T, Kanegee Y, Saito I, Toyoda Y: Transgenesis by adenovirus-mediated gene transfer into mouse zona-free eggs. *Nature Biotech* 14:982–985, 1996

Tyner AL, Eichman MJ, Fuchs E: The sequence of a type II keratin gene in human skin: conservation of structure among all intermediate filament genes. *Proc Nat Acad Sci USA* 82:4683–4687, 1985

Weiss RA, Eichner R, Sun T-T: Monoclonal antibody analysis of keratin expression in epidermal diseases, a 48- and 56-kdalton keratin as molecular markers for hyperproliferative keratinocytes. *J Cell Biol* 98:1397–1406, 1984

Winter H, Langbein L, Praetzel S, *et al*: A novel human type II cytokeratin, K6hf, specifically expressed in the companion layer of the hair follicle. *J Invest Dermatol* 111:955–962, 1998